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(54) Title: LIGHT SCATTER-BASED IMMUNOASSAY WITHOUT PARTICLE SELF AGGREGATION

(57) Abstract

A homogeneous immunoassay method for the simultaneous determination of one or more antibody, antigen or hapten analytes in a fluid sample, that comprises the quantification of the effect of said analytes on the statistical changes in a dimension of a light scatter pulse height distribution histogram of relatively large diameter monodisperse binding molecule-coated polymeric microspheres induced by the binding to said microspheres of polydisperse binding molecule-coated colloid metal particles of relatively small diameter. For simultaneous assays of multiple analytes, different diameter or refractive index microspheres are assigned to each analyte. The assay may be used in forward binding, displacement, inhibition, and competition type systems, with the direction of the change in histogram dimension depending on the system. A convenient dimension to measure is the normalized peak width of a graphical representation of the histogram. For simultaneous assays for multiple analytes, a monodisperse polymeric microsphere of unique diameter or refractive index is dedicated to each analyte, so as to generate multiple histograms, one for each analyte. Polydisperse binding molecule-coated colloid metal particles, when used in effective concentrations, will also serve as a scavenger means to reduce the interfering effects of non-specific substances in the analyte-containing fluid samples, particularly when said samples are of biological origin. An improved sheath flow cell for an FPA is also described.

LIGHT SCATTER-BASED IMMUNOASSAY
WITHOUT PARTICLE SELF AGGREGATION

BACKGROUND OF THE INVENTION

Field of the Invention

5   The invention relates broadly to homogeneous immunoassay methods for measuring an antigen, antibody or hapten analyte in a fluid sample by measuring light scatter signals from monodisperse particles in a flow particle analyzer. More particularly, the invention

10   relates to measuring changes in light scatter signals from binding molecule-coated monodisperse polymeric microspheres as the result of the analyte-mediated binding to such microspheres of binding molecule-coated polydisperse colloidal metal particles.

Description of the Prior Art

15   Antibodies, antigens and many haptens exhibit high affinities, not only for their complementary proteins, but also for certain solid surfaces such as those found in the wells of plastic microtiter plates, walls of

20   plastic test tubes, polymeric microspheres, and colloidal metal particles. Exploitation of these properties has led to a revolution in the field of diagnostic assay methods for the aforementioned analytes in fluid samples such as serum.

25   The ability to carry out antigen-antibody interactions on solid supports has greatly simplified

the separation of analyte-containing immunocomplexes
from unused reactants and interfering substances, such
as those often present in biological fluids. Such
systems are generally referred to as "solid phase
5   immunoassays" or "immunosorbent assays", and fall
within the genus of "heterogeneous immunoassays." While
the phase separation steps in heterogeneous
immunoassays are valuable in reducing interferences by
nonspecific binding substances that generally have an
10   adverse effect on the sensitivity of the assay method,
such assays are cumbersome and expensive, and are a
focal point for reliability problems in automated
systems.

     Additionally, such heterogeneous systems have the
15   additional disadvantage of requiring that one or
another member of the immunocomplex be labeled with a
molecule that can be easily quantified. Such molecules
are generally referred to as "reporter molecules" and
include radioisotopes (radioimmunoassays, RIA), enzymes
20   (enzyme-linked immunoassays generally coupled with a
chromophore, ELISA), fluorescent molecules
(fluorescence immunoassays, FIA), chemiluminescent
molecules(CIA), gold particles, photosensitive
molecules, and the like. For a review, see Kemeny,
25   D.M., et al., Immunology Today, 7: 67 (1986). Further,
because of the limited number of chromophores and
fluorophores available as receptor molecules, and the
extensive overlap of emission spectra of such
molecules, simultaneous assay of multiple analytes are
30   not suitable using these reporters. For example,
Cambridge Biotech's simultaneous EIA assays for C.
difficile Toxin A and Toxin B, HTLV-I and HTLV-II, and
HIV-1 and HIV-2, are not separable. The present
invention greatly simplifies separability of signals

from multiple analytes in a single reaction mixture.

"Homogeneous immunoassay" is the term applied to immunoassays in which no phase separation occurs. Such systems, which include binding protein-coated particle agglutination assays, are useful because they have fewer steps to automate, and automation is mechanically, fluidically and electrically simple. Examples of immunoassays requiring no phase separation steps include: latex microsphere agglutination, hemagglutination, and fluorescence depolarization assays. Examples of latex bead agglutination assays for single analytes are found in U.S. patent nos. 4521521, 4184849, 4279617, 4191739 and 4851329, and for multiple analytes in a single fluid sample in Hansen, copending U.S. patent application no. 883574. Nephelometric or turbidimetric automated systems for agglutination or fluorescence depolarization assays are simple, inexpensive to construct, and, unlike heterogeneous assays, do not require frequent maintenance of the complicated phase separation apparatus.

The presence of interfering substances in body fluids has, however, inhibited otherwise promising homogeneous immunoassay approaches from meeting the high sensitivity requirements of many medically important tests, such as are met by ELISA and RIA. For reviews of this problem see, for example, Masson et al., Methods in Enzymology, 74:115(1981) and Collett-Casssart et al., Clin Chem., 27:64 (1981). One important aspect of the present invention is that it is a homogeneous immunoassay that is free from non-specific interferences, at least to a sensitivity level of about 5×10^{-13} M. This level of sensitivity is two to three orders of magnitude greater than prior art homogeneous latex bead agglutination assays. See,

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e.g., alpha-fetoprotein (3 x 10^{-10} M, Collett-Cassart et
al. above), urinary HCG (6 x 10^{-11} M, Lentrichia et al.,
J. Immunol. Meth., 89:657(1986) (but exhibiting only an
87% correlation with RIA at analyte levels 20 times the
5   claimed sensitivity limit), and serum digoxin by a
fluorescence depolarization method (3 x 10^{-10} M, S. Wong
in D. Chan, ed., Immunoassay Automation, Academic
Press, 1992, p. 329).

Prior art approaches to eliminating or decreasing
10   the undesirable effects of non-specific interfering
substance on homogeneous immunoassays have been
generally unsatisfactory. These include: high dilution
of body fluid sample (Fritz et al., J. Immunol.,
108:110(1972), but this proportionally decreases
15   sensitivity; using antibody fragments (Masson, Id.),
but this approach is expensive and unpredictable; and,
use of special conditions of pH, ionic strength, and
buffer type, and/or addition of chelators or other
scavengers (Masson, Id.), but these introduce multiple
20   dependent factors that must be optimized for each
analyte, and can become prohibitively expensive and
cumbersome (Lim et al., J. Clin. Chem. Clin. Biochem.,
20:141(1982).

Other approaches to solving the non-specific
25   interference problem have included using IgG-coated
latex ultramicrospheres to inhibit non-specific
reactions in a latex sphere agglutination assay that
uses antibody fragments. The sensitivity of one such
agglutination method using a Coulter principle
30   electronic resistance flow particle analyzer with a 30
μm orifice was reported to be about 5 x 10^{-13} to 4 x 10^{-12} M. Sakai et al., Chem. Pharm. Bull., 37:3010 (1989).
The disadvantages of this approach is that the
additional reagent (non-specific, IgG coated

ultramicrospheres) has an incremental manufacturing, quality control and storage cost associated with it. The present invention removes this important disadvantage by combining the action of specific immunoreactivity with the action of improving specificity, all in one reagent. In addition, although the Coulter principle particle counter used by Sakai et al. yields quantitative results, the need for the small (30 μm) orifice in order to sense agglutination has the well known problem of clogging during agglutination reactions (Masson, Id.). The present invention uses a sheath flow particle analyzer with a 250-300 μm orifice, which eliminates clogging. However, in the Sakai et al. approach, the distribution of specifically agglutinated particles (dimers, trimers, etc) presents a problem in Coulter volume overlap if simultaneous assays of more than one analyte are attempted, a problem not encountered in the present invention as multimers are not formed and multiple simultaneous assays can be performed without algorithms to remove the problem of overlap.

Another problem encountered in prior art agglutination immunoassays is the need for agitation by mechanical mixers during the entire reaction period of reaction mixtures containing particles of one micron or greater (Masson, Id.), thus requiring stringent washing between samples to prevent carryover of samples. A further advantage of the present invention, insofar as automation is concerned, is that agitation of samples is not needed to complete the agglutination reaction during useful time frames.

Schutt et al., EP 0254430 and United States Patent No. 5017009, show a scattered total internal reflectance (STIR) assay method for an analyte in which

colloidal gold particles are used as a label for proteins that bind to a coated macroscopic plastic plate of optical quality. This immunoassay relies upon the detection of back scattered light from an

5   evanescent wave disturbed by the presence of a colloidal gold label brought to the interface by an immunological reaction. This evanescent wave is said to be the result of a totally internally reflected incident light wave. A disadvantage of this system is

10   that the expensive optical-quality plastic plate is not reusable and must be discarded after a single use.

There remains an important need for an immunoassay method for antigens, antibodies, and haptens in fluid samples that combines the mechanical simplicity and low

15   cost of particle agglutination homogeneous assays with the reduction in deleterious effects of interfering substances enjoyed by solid support based heterogeneous assays, and that does so with increased efficiency and scope when compared to prior art agglutination assays.

20   This need is now fulfilled by the invention described in detail below.

SUMMARY OF THE INVENTION

The invention relates to a novel immunoassay method for simultaneously detecting and quantifying the

25   concentration in a single fluid sample of one or more antigen, hapten or antibody analytes. The invention is based upon the serendipitous and unexpected observation that the binding of relatively small polydisperse binding molecule-coated colloidal metal particles to

30   the surface of relatively large monodisperse binding molecule-coated polymeric microspheres produces changes in the dimensions of light scatter pulse height distribution histograms of these polymeric

microspheres, and that these changes in histogram
dimension can be correlated with the concentration of
the analyte that induces the dimension changes.
Preferred light scatter is substantially low angle

5   forward light scatter or substantially right angle
light scatter. A convenient histogram dimension to
determine for this purpose is the normalized peak width
of a graphical representation of the histogram, that
is, the peak width at one-half peak height.

10   In a forward binding reaction (sandwich)
embodiment, a monodisperse immunocomplex is formed
between large monodisperse polymeric microspheres
coated with a first binding molecule, small
polydisperse colloid metal particles coated with a

15   second binding molecule, and an analyte that is
complementary to both binding molecules. The
dimensions of the light scatter pulse height
distribution histogram of the monodisperse particulate
immunocomplex are compared with control histogram

20   dimensions (obtained with monodisperse coated polymeric
microspheres in the absence of metal particles and
linking analyte tested before, after, or during the
analytical run), and the statistical increases in
histogram dimension due to the presence of the analyte

25   are correlated with the concentration of the analyte in
the fluid sample. In a displacement embodiment of the
invention, an immunocomplex reagent is first formed
between monodisperse polymeric microspheres coated with
the analyte and polydisperse colloidal metal particles

30   coated with an anti-analyte antibody, the light scatter
histogram dimensions of the immunocomplex is determined
before and after its exposure to the analyte, and the
statistical changes of the histogram dimension after
exposure to the analyte correlated with analyte

concentration in the fluid sample. In a competition embodiment of the invention, an analyte competes against anti-analyte antibody-coated polydisperse colloidal metal particles for binding to monodisperse polymeric microspheres coated with a first binding molecule complementary to the anti-analyte antibody. A reduction by analyte in the extent of immunocomplex formation between the microspheres and metal particle, reduces a dimension of the light scatter pulse height distribution histograms in proportion to the concentration of analyte in the fluid sample. In an inhibition reaction embodiment, the immunoassay relies upon the ability of analyte to inhibit the binding of anti-analyte-coated polydisperse metal particles to binding molecule-coated monodisperse polymeric microspheres.

In one aspect of this invention, there are disclosed details and scope of forward binding, displacement, competition and inhibition embodiments of the aforementioned inventive method.

In another aspect there are disclosed polymeric microspheres and colloid metal particles suitable for carrying out the method of the invention.

In still another aspect there are disclosed examples of the application of the inventive method to the estimation of specific analytes in biological fluid.

It is yet another aspect of the invention to disclose application of the inventive method to the simultaneous assay of multiple analytes in the same fluid sample in a single analytical run.

It is yet another object to disclose the concurrent use of coated metal particles, not only for analytical purposes, but also as scavengers that

advantageously remove interfering non-specific substances present in biological samples from immunoassay reaction mixtures.

These and other objects will become apparent by reference to the following detailed description of the preferred embodiments and the appended claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows polymeric microspheres and metal particles (A), and light scatter pulse height distributions before (B) and after (C) binding of metal particles to monodisperse polymeric particles. Figure 1(D) shows the simultaneous analysis of multiple analytes in a single sample by a combination of the present embodiments (histograms D1, D2, D3, D4).

Figure 2 shows (A) the normalized peak width parameters and (B) calibration (i.e., standard) curve for a forward binding (i.e., sandwich) assay.

Figure 3 shows a human serum TSH assay standard curve (A) and comparison with other methods (B).

Figure 4 shows a T4 standard curve (A) and comparison with other methods (B), in human serum.

Figure 5 (A) shows a latex-latex agglutination assay for IgE and (B) the correlation curve for the method of the invention.

Figure 6 shows the kinetics of binding of colloidal gold particles to latex microspheres brought about by analyte.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to novel immunoassay methods for antigen, antibody and hapten analytes in a fluid sample based on flow particle analysis. The methods take advantage of the serendipitous and unexpected

discovery that a statistical change occurs in measured
values of certain physical properties of a preparation
of relatively large monodisperse polymeric microspheres
when relatively small polydisperse metal particles bind
5   to the former microspheres in an immunochemical
reaction induced, proportionally to concentration, by
an analyte. Examples of particle properties that can be
monodispersed, as measured by flow particle analyzers,
are: the so-called Coulter-volume of insulating
10   particles in a given orifice diameter in an electrical
impedance flow particle analyzer; the fluorescent
emission by single particles when illuminated at a
given wavelength, also in an optical flow particle
analyzer; and, the light scatter pulse height
15   distribution histogram of monodisperse microspheres
substantially in a given direction when illuminated in
an optical flow particle analyzer. The last-named
property is used in the present novel method for
concurrently measuring one or more antigen, antibody,
20   or hapten analyte in a single fluid sample, a method
that is also capable of avoiding or reducing the
deleterious interfering effects of non-specific
substances in biological fluid samples, in prior art
agglutination immunoassays.
25   The optical flow particle analyzer (FPA) used in
the invention uses a sheath flow cell through which
passes a guided narrow sample stream, and light
scatter, preferably substantially low angle forward
light scatter or substantially right angle light
30   scatter, of an incident light beam, preferably a laser
beam, to sense and measure the degree of
immunochemically-induced complex formation between
relatively small polydisperse colloidal metal particles
and a population of relatively large monodisperse

polymeric microspheres. The method is based upon the unexpected discovery that the aforementioned polydisperse metal particles, which themselves do not scatter light under the conditions of the invention in

5   the region of the polymeric particles (even after the former are agglutinated by virtue of reaction with nonspecific binding components found often in sera), cause profound changes in the light scatter pulse height distribution histogram dimensions of the

10   monodisperse polymeric microspheres when the metal particles bind to the surface of the polymeric microspheres to form an immunocomplex. Where the polymeric microspheres are coated with a first complementary binding molecule and the metal particles

15   with another, a protein (i.e., antigen or antibody) or hapten analyte that is complementary to both first and second binding molecules will crosslink the metal particles to the polymeric particles, and will bring about a change in the dimensions of the light scatter

20   pulse height distribution histogram of the monodisperse polymeric microspheres compared to the control histogram dimensions obtained in the absence of metal particles and analyte. The aforementioned change in the histogram dimensions has been found to correlate

25   directly with the amount of the analyte present in the fluid sample.

       It is an important discovery that the aforementioned change in the histogram dimension occurs in the absence of any significant shift in the position

30   of the monodisperse polymeric microsphere light scatter pulse height distribution histogram, which indicates that self-aggregation of polymeric microspheres is not involved.

       The preferred dimension for monitoring changes in

the histogram is the peak width at one half peak height of the graphical representation of the light scatter pulse height distribution histogram. This dimension will be referred to in this specification as

5   "normalized peak width" or "NPW" (see Figure 2A for example). In the forward binding (sandwich) immunocomplex formation method described above, the change in histogram dimension is a widening of the NPW in the graphical representation and, consequently, an

10   increase in the coefficient of variation (CV) around the histogram mean.

It has also been discovered that measurement of statistical changes in the dimension of a light scatter pulse height distribution histogram can be the basis

15   for a quantitative determination of an analyte in a fluid sample in displacement-, competition- and inhibition-type immunoassay methods.

In a displacement embodiment, an immunocomplex reagent is prior-prepared by immunobinding polydisperse

20   colloid metal particles coated with an anti-analyte antibody to monodisperse polymeric microspheres coated with analyte. A base line determination of the light scatter pulse height distribution histogram of this reagent will show a dimension that resembles that which

25   results when the two types of particles are immunocomplexed, that is, a wide NPW if this is the dimension examined. When this reagent is mixed with a solution containing an analyte that is complementary to the binding molecule with which the metal particles are

30   coated, the metal particles will be displaced from the immunocomplex reagent in direct proportion to the concentration of analyte present, and the histogram dimension will return to that resembling control monodisperse polymeric microspheres, that is, the

dimensions of the histogram will be reduced, that is to say, the NPW (and CV) will be reduced. The statistical extent of dimension reduction, as reflected in the NPW, can be correlated by standard means to the
5   concentration of analyte in the fluid sample.

In a competition embodiment, binding molecule-coated monodisperse polymeric particles and analyte compete for binding to binding molecule-coated polydisperse metal particles. The NPW for the
10 polymeric particles is inversely related to the concentration of fluid sample being analyzed, that is to say, a high analyte concentration will "capture" a relatively larger fraction of the metal particles, leaving a relatively smaller fraction of metal
15 particles to bind to the polymeric microspheres. This will produce a relatively narrow histogram, that is, a small NPW. As in the previous two embodiments, the analyte may be an antigen, hapten or antibody, and it is well within the skill of the assayist, following the
20 guidelines provided by the invention, to select appropriate binding molecules with which to coat the microspheres and metal particles.

In an inhibition embodiment, the analyte-containing sample is incubated with anti-analyte
25 antibody-coated polydisperse metal particles and incubated for a finite time which does not necessarily include the time necessary to reach equilibrium, which may be on the order of hours. Analyte-coated monodisperse polymeric microspheres are then added and
30 incubation is continued for another finite time, which is of the order of minutes, during which period the analyte inhibits the binding of metal particles to the polymeric microspheres. Histogram dimensions are determined as above. High concentrations of analyte

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will produce narrow histogram peak widths as the result
of its sequestering of the metal particles; low
concentrations of analyte will produce the reverse.

As noted above, it is an important feature of the
5 embodiments of the invention that incubation mixtures
used need not be stirred during reaction periods. When
adding components of reaction mixtures, only brief
mixing (1-2 seconds) to produce homogeneity of the
mixture is required.

10   A variety of commercially available polymeric
particles may be used in this invention, although
highly preferred are uniform latex microspheres. Bangs,
L.B., Uniform Latex Particles, Seragen, Indianapolis,
1984. Although the term "latex", strictly speaking,
15 refers to the polyisoprene of which milk sap is
composed, the definition of this term has been
expanded, and will be used herein to include synthetic
polymers such as polybutadiene, polystyrene and the
like. Uniform latex microspheres of average diameter
20 ranging between 0.05 and 10 μm, preferably between
about 0.5 and about 5.0 μm, and having stable
hydrophobic surface groups to which proteins bind
strongly so as to produce stable hydrophilic colloidal
suspensions, are preferable diameters for use in the
25 invention, although diameters ranging up to about 100-
120 μm are available commercially. The 0.5 to 5.0 μm
diameter latex microspheres with highly monodispersed
diameters are available from Polysciences, Inc.,
Warrington, PA 18976 and from Interfacial Dynamics
30 Corp., Portland, OR 97220. The standard deviation of
diameters expressed as per-cent of the mean (i.e.,
coefficient of variation, or CV) are approximately 1%
to 2% for these commercial preparations, and it is
highly preferred that CV's of 2% not be exceeded.

The CV of the light scatter pulse height distribution histogram obtained from these spherical particles is a strong function of the relative diameter of the sample stream through the flow cell of the FPA

5   and the focal dimensions of the incident (e.g., laser) light. If the sample stream is relatively large, then particles can flow through different intensities of the incident light beam and yield an undesirably large CV of pulse heights, even though the CV of the particles

10 themselves is small. Therefore, it is essential that a narrow sample stream of constant dimensions be maintained in order to practice this invention optimally. Means for accomplishing this will be described below.

15   Although central bore diameters for the optical capillary flow cell ranging between 100 μm and 500 μm are suitable for purposes of the present invention, a central bore diameter of about 250 μm is preferred. It is preferred to center the fluid sample stream and to

20 confine it to a diameter range of about 3 μm to 10 μm. In a highly preferred system, this diameter amounts to about 1% to 3% of the width of the laser light beam. Under these conditions, CV values for light scatter pulse height distribution histograms of less than 2%

25 for monodisperse polymeric microspheres can be obtained, and are most preferred. As used in the present context "monodisperse" is taken to mean a population of polymeric microspheres that produce a low angle forward scattered light pulse height distribution

30 histogram with a CV of no greater than about 2%. When the sample stream diameter is not controlled so as to give a histogram CV of about 2% or less for non-immunochemically sensitized control monodispersed spherical particles, the sensitivity of the method is

adversely affected, and the symmetrical broadening or narrowing of the histogram are not readily observed.

A variety of fluid drive means can be used to achieve the aforementioned narrow fluid stream. These include stepped motor controlled syringe pumps and vacuum devices. Persons skilled in this art will recognize that the best mode would be one in which the stream diameter could be either directly or indirectly regulated during a measurement and controlled to the proper dimension range. It is also desireable to provide the flow cell with a stream centering means so as to confine the narrowed fluid sample stream as precisely as possible in the center of the sheath flow. To monitor both the narrowing and centering features, it is possible to monitor the sample stream before, after and during the times during which the immunoreacted particles are measured. This can be accomplished in either case with a control population of monodisperse polymeric microspheres of the same or different diameters as that of the analytical microspheres and that do not participate in any immunobinding reactions. Such control microspheres can serve as a component of a sentinel system that includes an electronic sensing means that senses departures from the desired CV value, and that feedback regulates both or either the stream narrowing means and stream centering means so as to achieve the desired CV value.

As noted above, to carry out the inventive method in the forward binding (sandwich) reaction embodiment, both the relatively large monodisperse microspheres and the relatively small polydispersed metal particles are coated with different complementary binding molecules. Where the analyte is an antigen or hapten, monodisperse

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polymeric microspheres are coated with a first
complementary anti-antigen or hapten analyte antibody,
which may be a monoclonal antibody directed to a first
epitope on the antigen. The polydisperse metal

5　　　particles are coated with a second anti-antigen analyte
antibody, which may be a monoclonal antibody directed
to a second epitope of the antigen analyte. In the
presence of antigen, a typical "sandwich" reaction
takes place, with the antigen crosslinking the smaller

10　　metal particles randomly on the surface of the larger
polymeric microspheres. When this occurs, and the
resulting particulate immunocomplexes are passed
through the optical flow cell described above, we have
found unexpectedly that the CV and standard deviation

15　　of the monodispersed light scatter pulse height
distribution histogram, as reflected in a graphical
representation of the histogram, produces peaks that
broaden approximately symmetrically about the peak
mean. We have also discovered that this broadening is

20　　directly and quantitatively related to the
concentration of the analyte in the fluid sample.

　　　　The principle of this embodiment of the invention
is shown in Figure 1. In Figure 1A are shown the
relative diameters of monodisperse latex microspheres

25　　(about 1.0 μm diameter) and the polydisperse colloidal
metal particles (about 20 to 120 nm in diameter). In
the preferred particle diameters described above, it
can be seen that the ratio of polymer microsphere
diameter to metal particle size ranges between 15-30:1.

30　　This characteristic provides the potential for a large
number of colloidal metal particles to be bound
randomly on each polymeric microsphere. The light
scatter pulse height distribution histogram for the
latex microspheres is shown in Figure 1B. The pulse

height signal from the latex microspheres is high, and
the width of the peak representing the histogram is
very narrow. As is evident, the small amount of light
scatter from self agglutinated metal particles does not

5   interfere, i.e., does not overlap, with the histogram
produced by the microspheres. After analyte-mediated
binding of the metal particles to the latex
microspheres, a broadening of the latex histogram peak
occurs (Figure 1C) in a forward binding reaction. Even

10   when multiple analytes in a single sample are analyzed
simultaneously by a combination of the present
embodiments (histograms D1, D2, D3 and D4 in Figure
1D), there is no overlap with the signal generated by
unbound gold particles.

15   Methods for producing stably adsorbed proteins on
latex microsphere surfaces are described generally in
Seaman, G.V.F., ed., Latex Based Technology in
Diagnostics, Health & Science Communications,
Washington, D.C. 20005, 1990, which is incorporated by

20   reference.

We have discovered that this invention may also be
carried out in a displacement embodiment. To carry out
the invention in this mode, the two types of particles
are first immunochemically sensitized by incubation

25   separately with complementary binding molecules to form
a two component immunocomplex reagent. In one
embodiment, the large polymeric microspheres are coated
stably with analyte. The metal particles are coated
with a binding molecule that is complementary to the

30   analyte. The two suspensions are mixed to form a
reagent that may be stored prior to use. This reagent
is then mixed with the analyte-containing fluid sample.
The analyte displaces a portion of the bound metal
particles from the polymeric microspheres by virtue of

immunocomplex formation. This displacement results in a decrease in the dimension of the light scatter pulse height distribution histogram of the aforementioned immunocomplex reagent. This decrease is proportional to the concentration of analyte present in the fluid sample.

In a competition-type embodiment, an analyte and an analyte-coated polymeric microsphere compete for binding to anti-analyte-coated metal particles. After an appropriate incubation period, the suspension is subjected to FPA and histograms determined. A control consists of the coated polymeric particles alone.

As noted above, in an inhibition embodiment, analyte is incubated with anti-analyte binding molecule-coated polydisperse metal particles for a finite period of time, e.g., 5-30 minutes, during which period the analyte binds to and "sequesters" a portion of the metal particles. Unbound metal particles are free to bind to polymeric microspheres coated with a binding molecule (which may be a carrier binding molecule that contains analyte) that are then added and incubated. FPA of control and experimental monodisperse polymeric microspheres after a brief period, e.g., 5-10 minutes, of incubation will reveal the amount of unbound metal particles which, in turn, is inversely related to the concentration of analyte.

While it has been convenient to describe the forward binding (sandwich) reaction, inhibition, displacement and competition embodiments of the invention in terms of an antigenic or hapten analyte and antibody-coated particles, it should be understood that the scope of this invention includes assays in which the analyte is an antibody present in, for example, human serum and the like. In such assays

carried out in the forward binding mode, both types of
particles may be coated with the antigen that is
complementary to the analyte or, optionally, one type
of particle may be coated with the antigen and the
5   other with a second antibody directed against the
antibody analyte. In this mode, in the presence of the
antibody analyte, the small polydisperse metal
particles will complex with the monodisperse polymeric
particles, and the abovedescribed statistical changes
10   in the light scatter pulse height distribution
histogram measured.

The foregoing has described assaying for a single
analyte in a fluid sample. It is within the scope of
this invention to analyze concurrently multiple
15   analytes in the same fluid sample without the need to
split the sample for multiple assays as must be done in
prior art methods. This may be accomplished by
assigning to each analyte to be determined a polymeric
microsphere of a unique diameter and/or refractive
20   index and a type of binding reaction. The coatings on
the different size microspheres will, of course, be
determined by the type of assay system to be assigned
to the analyte, e.g., forward binding, inhibition,
displacement or competition. The assay systems for the
25   multiple analytes may be the same or different. This
embodiment of the invention relies upon the electronic
components of the FPA to monitor the light scatter
signals produced by each differently sized polymeric
microsphere. Electronic systems for monitoring each
30   size of microsphere are disclosed in copending United
States patent application serial no. 883,574, which is
incorporated by reference. Briefly, signals from light
detectors that receive the light scatter signals from
polymeric microspheres may be analyzed by either of two

analytical systems. In a preferred analytical system
which is software based, pulses from the light scatter
detector are fed to an analog-to-digital converter
which samples the peak height of each pulse and passes
these peak height values to a computer which sorts the
peak height values by size and then arranges them in a
histogram, which may be a smoothed histogram, one for
each analyte.

Binding molecule-coated polymeric microspheres may
be purchased commercially (Polysciences, Inc.) or
prepared as described in Seaman, 1990, above. In a
typical coating procedure, suspensions of microspheres
are incubated with a buffered (pH 7 to 8) solution of a
first binding molecule at a concentration and for a
period of time (typically, 0.5 to 16 hrs) sufficient to
reach equilibrium binding of the molecule to the
microspheres. Coated microspheres are recovered by
brief centrifugation, and nonspecific binding sites
blocked by a brief (e.g. 15 min) exposure to a solution
of an inert protein (e.g., nonfat dry milk solids or
serum albumin). Coated microspheres are then washed at
least three times with an at least 4-fold volume of
cold storage buffer. Any storage buffer that provides
stability to the microsphere suspensions on storage may
be used. Typical storage buffers are 0.5% BSA-0.1% NaN_3
in 0.154 M NaCl, pH 7.4, or 0.1% BSA-0.01% NaN_3 in 10 mM
HEPES buffer, pH 7.5.

The polydisperse metal colloids may be of metals
and metal compounds, such as metal oxides, metal
hydroxides and metal salts. Examples include the
metals gold, platinum, silver and copper; gold is
highly preferred. Methods of production of colloidal
gold of the desired range of particle diameters, and
methods for coating metal particles with proteins, are

22

described generally in Roth, J., "The Colloidal Gold
Marker System for Light and Electron Microscopy
Cytochemistry", in Bullock, G.R. et al., <u>Techniques in
Immunochemistry</u>, 2: 217 (19), in Horisberger, M., <u>SEM</u>
5   11: 9 (1981), in Weiser, H.B., Inorganic Colloid
Chemistry, J. Wiley, N.Y. 1931, p. 1, in Leuvering,
J.H.W., United States patent no. 4,313,734, and in
Frens, G., <u>Nature, Physical Science</u>, 241:20 (1973), all
of which are incorporated by reference.

10   Polydisperse colloidal gold particle suspensions
may be obtained from E-Y Laboratories, Inc., San
Matteo, CA 94401 or prepared as described in the above-
cited references. In a preferred method, polydisperse
colloidal gold particles of particle size 10 nm to 120
15   nm diameter are produced by the method of Frens (1973)
above. Although this method produces a relatively
broad range of gold particle sizes, the dimensions of
the range is not a critical factor in the present
invention. As noted above, gold particles of such
20   sizes do not produce measurable and interfering light
scatter under the conditions of the invention. Within
the present context, therefore, by "polydisperse metal
particles" is meant a population of colloidal metal
particles whose diameters range between 20 nm and 120
25   nm.

In one method of coating gold particles with
antibody, the above-described solution of gold particle
is titrated to pH 7.5 using K_2CO_3. The coating
molecule, e.g., antibody is dissolved in 10 mM HEPES
30   buffer, pH 7.5, containing 0.02% BSA as a stabilizer,
and one-tenth volume is added to the gold particle
solution. After 60 mins of mixing, a one-tenth volume
of 0.1% non-fat dried milk solids is added in order to
block nonspecific binding sites. The particles are

washed three times by centrifugation in the storage
buffer (10 mM HEPES, pH 7.5, 0.1% BSA, 0.01% NaN₃, 1%
mannitol) and used immediately or stored at 4°C. Other
methods for coating gold particles are described in
5   Leuvering above, which is incorporated by reference.

Using the present invention, highly sensitive
assays for a multi-epitopic, high molecular weight
antigen such as thyroid stimulating hormone (TSH) may
be carried out by either passively or covalently
10   binding appropriately complementary polyclonal or
monoclonal antibodies to the colloidal metal (e.g.
gold) particles and polymeric (e.g. latex)
microspheres. The exact method of protein binding is
not critical provided that the immunological integrity
15   of the protein is not compromised, and that the binding
is stable under the conditions of the assay.
Particularly preferred for this assay are 1.62 μm
diameter latex microspheres with a CV of about 2%
(Interfacial Dynamics Corp.). Colloidal gold particles
20   with polydispersed sizes in the range of 50 to 80 nm,
as determined by electron microscopy, made as described
above, are particularly preferred.

As noted above, I have found that colloidal metal
particles, when used at high densities relative to
25   polymeric microspheres such as from 2 to 100,000 to 1,
act as "scavengers" for nonspecific interfering
substances commonly found in fluid samples of
biological origin, such as human serum. That is to
say, the metal particles not only provide the basis for
30   the quantitative immunoassay of the invention, but also
reduce the deleterious effects of interfering
substances. Although the useful particle ratio in this
regard is very broad, metal particle to polymeric
microsphere ratios of the order of 1000 to 10000:1 are

preferred.

The following examples are provided merely to illustrate several embodiments of the invention, and are not intended to delimit the scope of the invention

5   which is encompassed by the specification and included claims.

EXAMPLE 1

FORWARD (SANDWICH) BINDING REACTION ASSAY FOR SERUM TSH

Polydisperse colloidal gold particles (50-80nm)
10   were coated with a first anti-TSH monoclonal antibody
(BioDesign International, Inc., Kennebunkport, ME
04046) as follows. A suspension of colloidal gold
particles was brought to pH 7.5 using 0.2 M potassium
carbonate. To this suspension was added a 0.1 volume
15   of the coating antibody diluted in 10 mM HEPES-0.02%
BSA, pH 7.5. After mixing for 1 hr, a 0.1 volume of a
solution of non-fat dried milk solids were added with
mixing. The particles were washed three times by
centrifugation in 10 mM HEPES buffer (pH 7.5)
20   containing 1% BSA, 0.01% NaN$_3$ and 1% mannitol.

Polystyrene (latex) beads of 1.62 μm diameter
(approximate diameter CV of 2.0%) were coated with a
second anti-TSH monoclonal antibody as described in
Hansen, United States patent application serial no.
25   883,574, which is incorporated herein by reference.

Coated gold particles were mixed with coated latex
particles in a ratio of 10,000 to 1 to form Reagent A.
Reagent A was added to TSH-containing serum in a volume
such that the final latex microsphere density was about
30   2 x 10^6/mL, and the serum was 40%. After 60 minutes of
incubation at room temperature, the mixture was
measured in a sheath FPA as described above using low
angle forward light scatter. Normalized peak width

units ("NPW" in Figure 2) as a function of analyte TSH
concentration in the serum over the concentration range
of 0 to 1.2 μIU/mL is shown in Figure 3.   The
sensitivity of this assay was about 5 x 10^{-13} M in serum.

<div style="text-align: center;">EXAMPLE 2</div>

<div style="text-align: center;">INHIBITION ASSAY FOR THYROXINE (T4)</div>

Monodisperse latex microspheres (1.62 μm diameter,
Interfacial Dynamics Corp.) were coated with human
thyroglobulin (Calbiochem Inc.) as follows to form
Reagent A.   The microspheres were incubated overnight
with antigen thyroglobulin in 10 mM HEPES buffer, pH
7.5.   Microspheres, recovered by centrifugation, were
washed with HEPES buffer containing BSA and NaN$_3$ as
described above, and stored in the same buffer containg
mannitol.

Polydisperse colloidal gold particles (50 - 80 nm
diameter) were coated with an antibody to T4 as follows
to form Reagent B.   One-tenth volume of 10 mM HEPES
buffer (pH 7.5) containing antibody and BSA was added
to the gold particles.   For the T4 assay, an IgG-
purified polyclonal antibody specific to T4 (OEM
Concepts) was used for coating.   Coated particles were
then post-coated with a solution of non-fat dry milk
powder to block nonspecific binding sites.   Particles
were recovered by centrifugation and washed with 10 mM
HEPES buffer (pH 7.5) containing BSA and NaN$_3$.   Washed
particles were stored to the same buffer, to which
mannitol was added as a stabilizer.   Coated gold
particles were diluted into an assay buffer containing
50 mM glycine (pH 9), 10 mM EDTA, 0.01%
aminonaphthosulfonic acid (Sigma Chem. Co., St. Louis,
MO), 0.1% BSA, 0.3 M KI and 0.01% NaN$_3$.

Serum samples containing T4 were incubated with
Reagent B for 30 minutes, and then Reagent A was added

to deliver a microsphere density of 2 x 10^7/mL. After an additional 30-minute incubation, samples were analyzed in the FPA for histogram peak width determination using low angle forward light scatter.

5   Serum T4 inhibits the binding of gold particles with latex thyroglobulin. High concentrations of serum T4 produced narrow histogram peak widths, and low concentrations of serum T4 produced the converse (Figure 4A). The curve in Figure 4A was generated

10   using calibrator sera (Biomerica, Inc., San Luis Obispo, CA) and served as the standard curve for further comparisons with standard EIA methods, i.e., the EMIT method (Syva Corp., Palo Alto, CA). The correlation data are shown in Figure 4B. The

15   correlation was > 95% over the analyte concentration range shown.

EXAMPLE 3

SIMULTANEOUS ASSAY OF TSH AND T4

IN THE SAME FLUID SAMPLE

20   The TSH and T4 immunoassays described in Examples 1 and 2 were combined in order to demonstrate that multiple analytes may be analyzed in the same fluid sample. The T4 and TSH assays were carried out with monodispersed latex microsphere populations having mean

25   diameters of 0.95 μm and 1.62 μm, respectively. The final serum concentration was 15%, the gold particle concentration was 1 x 10^{10}/mL, and the latex microsphere concentrations were 1 x 10^8/mL for T4 and 5 x 10^6/mL for TSH.

30   Reactions were carried out in the following sequence. The TSH-assay reaction was performed as described in Example 1. After 30 minutes of this reaction, Reagent B of Example 2 was added. Twenty-

five minutes thereafter, Reagent A of Example 2 was added and the mixture incubated for five minutes. FPA analysis was then performed.

5   When midrange concentrations of the analytes were assayed with the FPA system and methods of the present invention, the results were 4 μg/mL for T4 and 2 μIU/ml for TSH. When a sample with no T4 and a TSH midrange level of 2 μIU/mL was assayed by the present method, the measured T4 value was zero, and the measured value

10   for TSH was 2 μIU/mL ± 10%. With a sample with a known low value for TSH (0.3 μIU/mL) and a known midrange value of 4 μg/mL for T4, the present method correlated within 10% with the known values.

This experiment demonstrates the way in which the

15   present invention permits simultaneous assays can be performed with separable results, and with no detectable cross-interference between assays in a single run.

EXAMPLE 4

20   <u>COMPARISON OF THE PRESENT METHOD WITH A STANDARD LATEX-LATEX AGGLUTINATION METHOD FOR IgE DETERMINATION</u>

In order to demonstrate the efficacy of the present invention in suppressing the adverse effects of non-specific binding, an assay for human IgE was

25   conducted in two formats. In the first format, ordinary latex microsphere-latex microsphere agglutination was carried out with anti-IgE polyclonal antibodies passively coated on the latex particles, according to the method of Masson et al., above, in

30   which the disappearance of monomeric particles is monitored. The latex particle diameter was 1.62 μm. The second format consisted of the forward binding reaction embodiment of the present invention.

To coat gold particles, a suspension of gold particles was titrated to pH 7.5 with 0.2 M K_2CO_3. To this suspension was added a one-tenth volume of mouse monoclonal anti-human IgE (Biodesign International,

5   Inc., Kennebunkport, ME, 04046) in 10 mM HEPES buffer, pH 7.5, containing 0.02% BSA. After 60 minutes of mixing, a one-tenth volume of 0.1% non-fat dried milk solids solution was added. The particles were isolated by centrifugation washed three times with the same

10   buffer, then stored in a storage buffer consisting of 10 mM HEPES, 0.01% BSA, 0.01% NaN_3, 1% mannitol.

To coat latex microspheres, 1.62 μm latex microspheres (Interfacial Dynamics Intnl., Portland, OR 97220) were diluted to a density of 0.5% in 10 mM

15   HEPES, pH 7.5, the suspension was incubated overnight at 4°C with a 100 μg/mL solution of affinity-purified goat anti-human IgE, microspheres were isolated by centrifugation and washed three time with HEPES buffer, and finally stored in 10 mM HEPES, 0.1% BSA, 0.01% NaN_3,

20   1% mannitol.

For assay, latex and gold particles were diluted to 1×10^8/mL and 2×10^{10}/mL, respectively, in assay buffer. A 50 μl aliquot of analyte serum sample was added to 450 μl of the particle mixture, and mixed by

25   vortexing; the final serum dilution was 10%. After 15 minutes of incubation at 23°C, the reaction mixture was subjected to FPA analysis. The assay buffer was 0.05 M glycine 0.1% BSA, 0.3 M KI, 0.01% NaN_3, pH 9.5.

A commercial ELISA IgE assay method for serum

30   IgE(Ventrex Laboratories, Portland, ME) served as the reference standard for both formats. The correlation curve for the latex-latex agglutination format is shown in Figure 5A and that for the method of the invention in Figure 5B. It is clear from the data that the

correlation is poor and that the sensitivity is limited for the latex agglutination method, likely due to the influence of interfering substances in the analyte serum sample. In sharp contrast, the latex

5   microsphere-gold particle method of the present invention shows an excellent correlation with the reference method (Figure 5B).

EXAMPLE 5

KINETICS OF HISTOGRAM PEAK WIDTH BROADENING

10   The assay of Example 1 was carried out in a manner that permitted low angle forward light scatter FPA analyses of the reaction mixture (containing 6 μIU/mL TSH) at intervals.

In the absence of analyte (-O-, Figure 6), coated

15   gold particles did not bind to coated latex microspheres, and there was no change in histogram dimension. However, in the presence of analyte (-□-, in Figure 6) binding was detected from the first time point (2 min.), as reflected by a broadening of the

20   histogram derived from the monodisperse latex microspheres. This broadening continued at a linear rate for the first 20 minutes of reaction, and reached a plateau at about 100 minutes.

I CLAIM:

1.   A polymeric particle light scatter-based
immunoassay method for simultaneously measuring one or
more antibody, antigen or hapten analytes in a single
fluid sample essentially without self-aggregation of
5   said polymeric particles, comprising the steps of:
            a)   combining with said fluid sample for
each said analyte first binding molecule-coated
monodisperse polymeric microspheres of unique diameter
or refractive index, such that the light scatter pulse
10   height distribution histogram obtained in an optical
sheath flow particle analyzer for each said unique
diameter or refractive index microsphere is resolvable
from all other unique diameter microspheres, and second
binding molecule-coated colloidal metal particles;
15   wherein said first and second binding molecules are
selected such that the extent of binding of the
colloidal metal particles to the polymeric microspheres
is dependent upon the amount of analyte in the fluid
sample;
20   b)   measuring, in an optical sheath flow
particle analyzer equipped with a regulatable means for
producing a narrow fluid reaction mixture stream, a
regulatable means for centering said narrow stream
flowing through an optical capillary sheath flow cell,
25   a source of incident light, and a means for measuring
the scatter of said incident light induced by said
microspheres, the effect of each said analyte on the
statistical change in the dimension of light scatter
pulse height distribution histograms of first binding
30   molecule-coated monodisperse polymeric microspheres
that are induced by the binding to said microspheres of
second binding molecule-coated polydisperse colloidal

metal particles; and

       c)   correlating said analyte effect with the
35   amount of each said analyte in said single fluid
sample.

       2.   A method of claim 1, wherein said incident
light scatter is substantially low angle forward light
scatter.

       3.   A method of claim 1, wherein said incident
light scatter is substantially right angle light
scatter.

       4.   A method of claim 1, wherein the binding of
said second binding molecule-coated polydisperse
colloidal metal particles to said first binding
molecule-coated polymeric microspheres is increased by
5   said analyte, and said increase results in an increase
in said dimension of said histogram.

       5.   A method of claim 1, wherein the binding of
said second binding molecule-coated polydisperse
colloidal metal particles to said first binding
molecule-coated polymeric microspheres is decreased by
5   said analyte, and said decrease results in a decrease
in said dimension of said histogram.

       6.   A method of claim 1, wherein said histogram
dimension comprises a normalized peak width of a
graphical representation of each said histogram.

       7.   A method of claim 1, further comprising the
step of providing a ratio of said metal particles to
said polymeric microspheres effective to reduce
interference in said immunoassay by nonspecific binding
5   substances.

       8.   A method of claim 7, wherein said ratio
ranges between 2 and 100,000 to 1.

       9.   A method of claim 7, wherein said ratio
ranges between 1,000 and 10,000 to 1.

10.   A method of claim 1, wherein said monodisperse polymeric microspheres comprise uniform latex microspheres.

11.   A method of claim 1, wherein said microspheres comprise microspheres of average diameter ranging between about 0.02 and about 100 μm.

12.   A method of claim 1, wherein said microspheres comprise microspheres of an average diameter ranging between about 0.05 and about 10.0 μm.

13.   A method of claim 1, wherein said microspheres comprise microspheres of an average diameter ranging between about 0.5 and about 5.0 μm.

14.   A method of claim 1, wherein said colloidal metal particles are selected from the group consisting of gold, platinum, silver and copper particles.

15.   A method of claim 1, wherein said colloidal metal particles are gold particles.

16.   A method of claim 1, wherein the average diameter of said polydisperse metal particles ranges between about 20 nm and about 120 nm.

17.   A method of claim 1, wherein the average diameter of said polydisperse metal particles ranges between about 50 nm and about 80 nm.

18.   A method of claim 1, wherein the diameter of said regulatable narrow sample stream ranges between about 3 μm and 10 μm and produces a coefficient of variation around a graphical representation of a

5   histogram mean of no greater than about 2% for control, non-immunochemically sensitized monodisperse polymeric microspheres.

19.   A method for detecting an analyte in a fluid sample by analyte-mediated binding reaction comprising the steps of:

        a)   placing in a reaction vessel said first

5   binding molecule-coated monodisperse polymeric

       microspheres, said second binding molecule-coated

       polydisperse colloidal metal particles, and said fluid

       sample containing said analyte for a period of time

       effective to form a reaction product consisting

10   essentially of a monodisperse immunocomplex between the

       three components;

              b)   analyzing said monodisperse immunocomplex

       in said flow particle analyzer;

              c)   measuring the dimensions of the light

15   scatter pulse height distribution histogram of said

       monodisperse immunocomplex;

              d)   comparing said dimensions in c) with the

       dimensions of a control reaction mixture composed of

       monodisperse polymeric microspheres analyzed in the

20   absence of analyte or metal particles, to determine the

       statistical change in said histogram dimension in c);

       and,

              e)   relating said statistical change in said

       histogram dimension to the concentration of said

25   analyte in said fluid sample.

              20. A method of claim 19, wherein said analyte is

       an antigen or hapten, said first binding molecule

       comprises a first complementary anti-analyte antibody,

       and said second binding molecule comprises a second

5   complementary anti-analyte antibody.

              21. A method of claim 19, wherein said analyte is

       an antibody, and said first and said second binding

       molecules are each an antigen or hapten complementary

       to said analyte antibody, or, optionally, one binding

5   molecule comprises an antigen complementary to said

       analyte antibody and the other binding molecule

       comprises a second antibody directed against said

       analyte antibody.

22.   A method of for detecting an analyte in a
fluid sample by analyte-mediate displacement reaction
comprising the steps of:

     a)   mixing analyte-coated monodisperse
polymeric microspheres and complementary binding
molecule-coated polydisperse colloid metal particles
or, optionally, analyte-coated metal particles and
complementary binding molecule-coated polymeric
microspheres, so as to form a monodisperse
immunocomplex reagent;

     b)   mixing said monodisperse immunocomplex
reagent with a fluid sample containing said analyte for
a period of time sufficient for said analyte to
displace a portion of said metal particles from said
monodisperse immunocomplex;

     c)   determining the dimensions of the light
scatter pulse height distribution histograms for said
monodisperse immunocomplex both before and after
displacement with analyte in step b); and,

     d)   relating the statistical changes in said
histogram dimensions to the concentration of said
analyte in said fluid sample.

23.   A method of claim 22, wherein said analyte is
an antigen or hapten.

24.   A method of claim 22, wherein said analyte is
an antibody, and said complementary binding molecule is
the corresponding antigen.

25.   A method for detecting an analyge in a fluid
sample by a competition reaction comprising the steps
of:

     a)   mixing anti-analyte antibody-coated
polydisperse metal particles, analyte, antigen or
hapten-coated monodisperse polymeric microspheres, and
a fluid sample containing analyte antigen or hapten for

a period of time sufficient for competition
immunocomplexes to form;

10   b)   determining the dimensions of the light
scatter pulse height distribution histograms for said
coated monodisperse polymeric microspheres before and
after said competition immunocomplex formation; and

         c)   relating the statistical changes in said
15 histogram dimensions to the concentration of said
analyte in said fluid sample.

     26.   A method for detecting an analyte in a fluid
sample by a competition reaction comprising the steps
of:

         a)   mixing antigen or hapten-coated
5 polydisperse metal particles, fluid sample containing
an analyte antibody, and monodisperse polymeric
microspheres coated with a binding molecule
complementary to said antigen or hapten for a period of
time sufficient for competition immunocomplexes to
10 form;

         b)   determining the dimensions of the light
scatter pulse height distribution histograms for said
coated monodisperse polymeric microspheres before and
after said competition immunocomplex formation; and

         c)   relating the statistical changes in said
15 histogram dimensions to the concentration of said
analyte in said fluid sample.

     27.   A method for detecting an analyte in a fluid
sample consisting of an inhibition reaction, comprising
the steps of:

         a)   coating monodisperse polymeric
5 microspheres with a first binding molecule that is or
is conjugated to analyte to form a first reagent;

         b)   coating polydisperse metal particles
with a second binding molecule that is complementary to

analyte to form a second reagent;

10   c)   incubating an analyte-containing fluid
sample with said second reagent to form a polydisperse
immunocomplex;

          d)   incubating said polydisperse
immunocomplex with said first reagent to form a

15   monodisperse immunocomplex between said microspheres
and that fraction of metal particles not bound to
analyte;

          e)   analyzing said monodisperse
immunocomplex in said flow particle analyzer;

20   f)   measuring the dimensions of the light
scatter pulse height distribution histogram of said
monodisperse immunocomplex;

          g)   comparing said dimensions in f) with a
control reaction mixture composed of coated

25   monodisperse polymeric microspheres in the absence of
analyte or metal particles to determine the statistical
change in said histogram dimension in f); and

          h)   relating said statistical change in said
histogram dimension to the concentration of said

30   analyte in said fluid sample.

          28.   A method of claim 27, wherein said analyte is
a hapten or antigen and said second binding molecule is
an anti-hapten or anti-antigen antibody.

          29.   A method of claim 27, wherein said analyte is
an antibody and said second binding molecule is
complementary to said antibody.

          30.   A method for detecting an analyte in a fluid
sample wherein more than one analyte is measured in a
single fluid sample, comprising the steps of:

          a)   assigning to each said analyte a forward
5   binding, displacement, inhibition, or competition
reaction;

b) selecting for each said analyte a unique first binding molecule-coated monodisperse polymeric microsphere and unique second binding molecule-coated polydisperse colloidal metal particles;

c) carrying out all said reactions in the same reaction vessel;

d) measuring the statistical change in the dimension of each unique light scatter pulse distribution histogram produced by each said reaction; and,

e) relating each said histogram dimension change to the concentration of each said analyte.

31. A method of claims 19-30, inclusive, wherein said histogram dimension is the normalized peak width of a graphical representation of said histogram.

32. A mercantile test kit for measuring an antibody, antigen or hapten analyte in a single fluid sample which comprises in separate container means:

a) monodisperse latex microspheres of average diameter 0.5 μm to 10.0 μm coated with a binding molecule, said monodisperse latex microspheres having a coefficient of variation around a mean of no greater than about 2%; and,

b) polydisperse colloid gold particles of average diameter of 20 to 120 nm coated with a binding molecule; and, optionally,

c) a reagent comprising a monodisperse immunocomplex between coated monodisperse latex microspheres and coated polydisperse gold particles.

33. In a flow particle analyzer capable of detecting and measuring a light scatter pulse height distribution histogram produced by monodisperse polymeric microspheres contained within a fluid sample stream flowing through an optical sheath flow cell of

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said analyzer, the improvement that comprises providing
for said optical sheath flow cell: a regulatable fluid
drive narrowing means for narrowing said fluid sample
stream passing through said cell; a regulatable

10   centering means for centering said narrowed fluid
sample stream through said cell; a sensing means for
monitoring the histogram dimension of control
monodisperse polymeric microspheres; and, a feedback
means for controlling said stream narrowing means and

15   said stream centering means so as to maintain said
control histogram dimension within desired limits.

34. An analyzer of claim 33, wherein said
narrowing of said fluid sample stream is to a diameter
ranging between about 3 μm to about 10 μm or between
about 1% and 3% of the width of a beam of incident
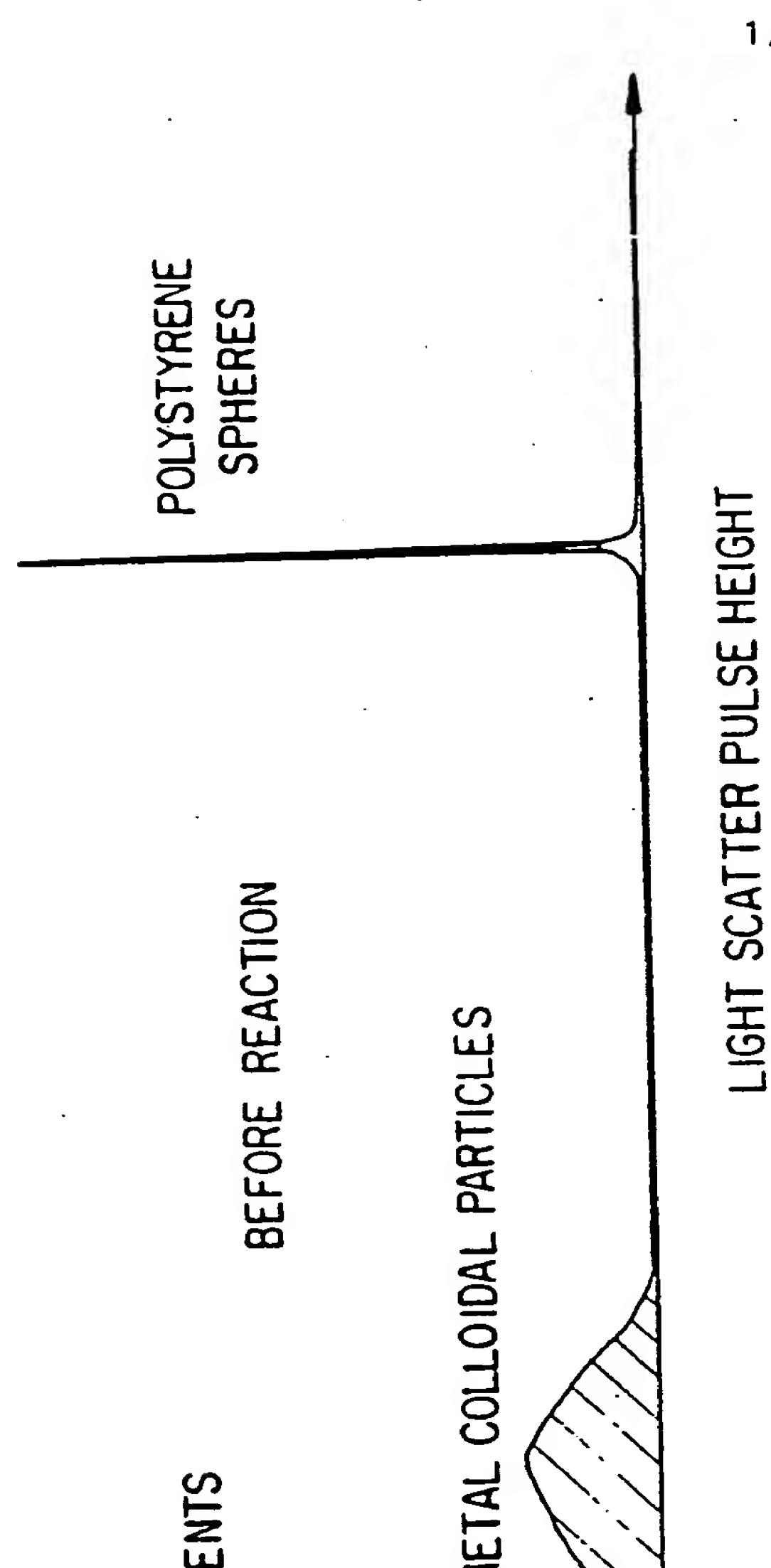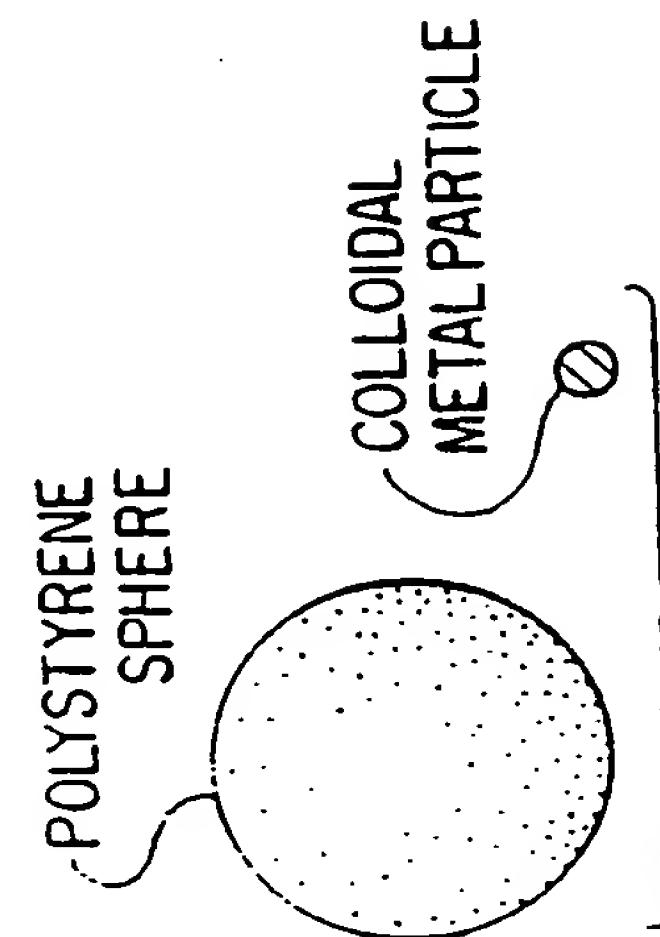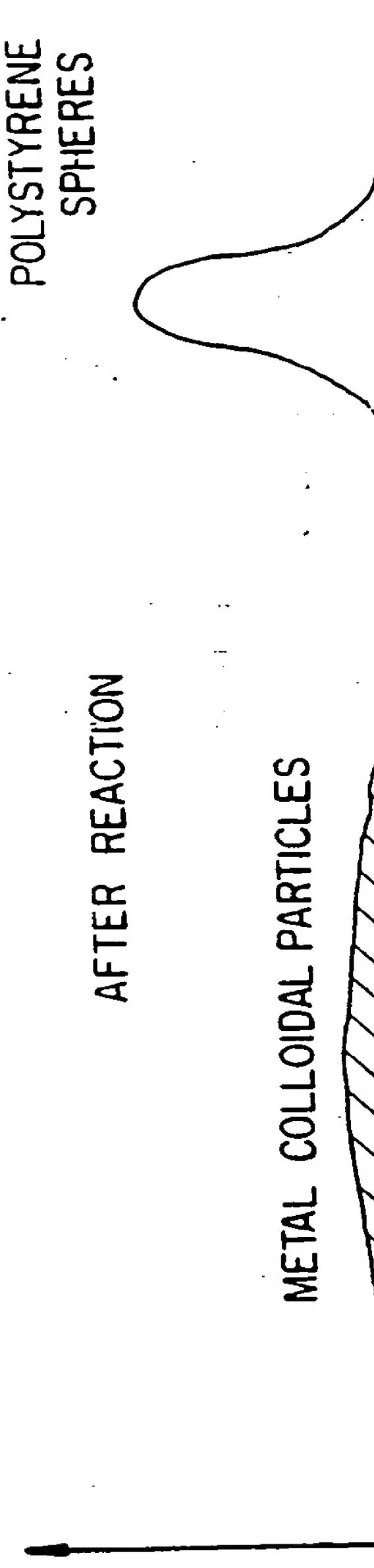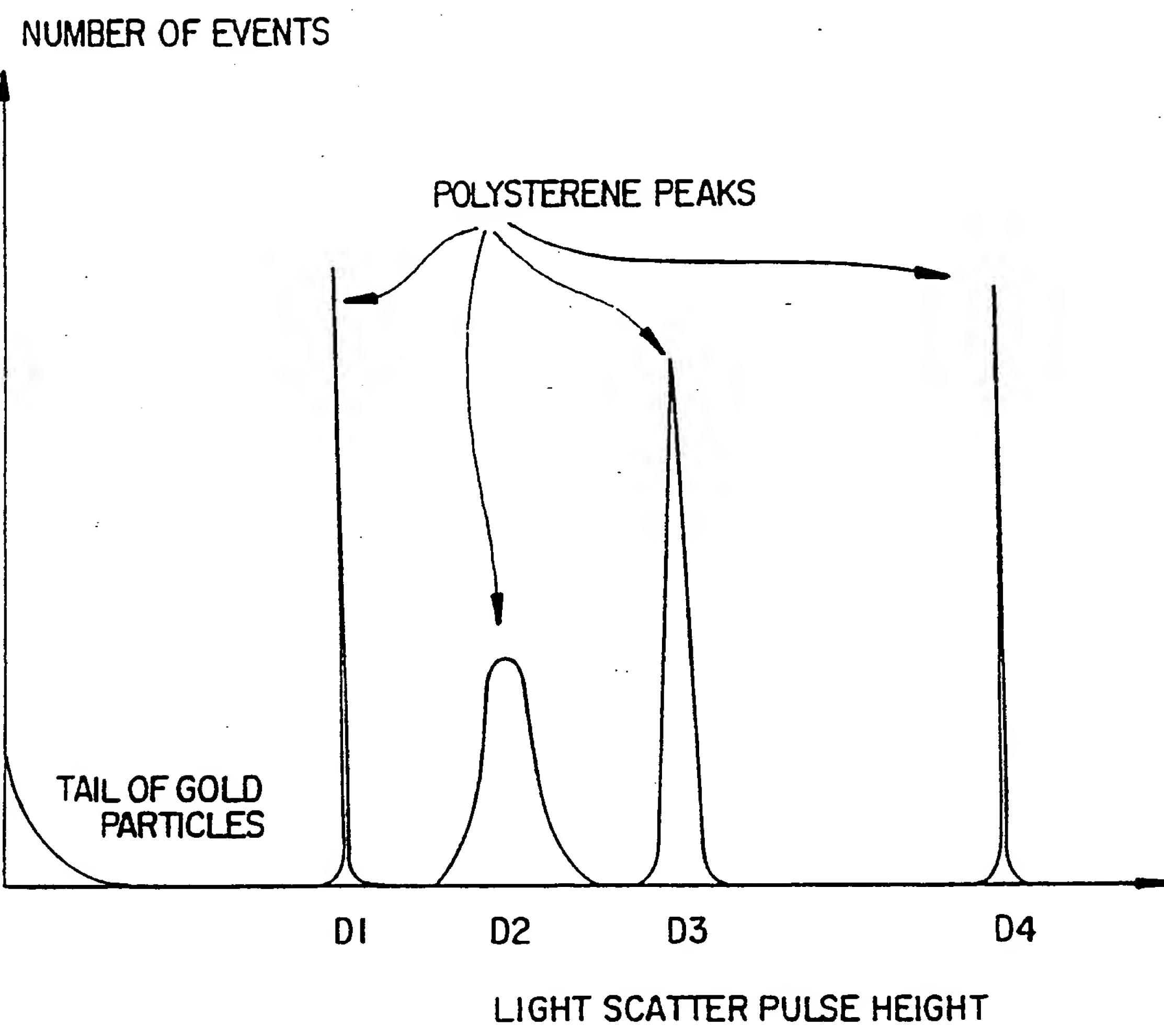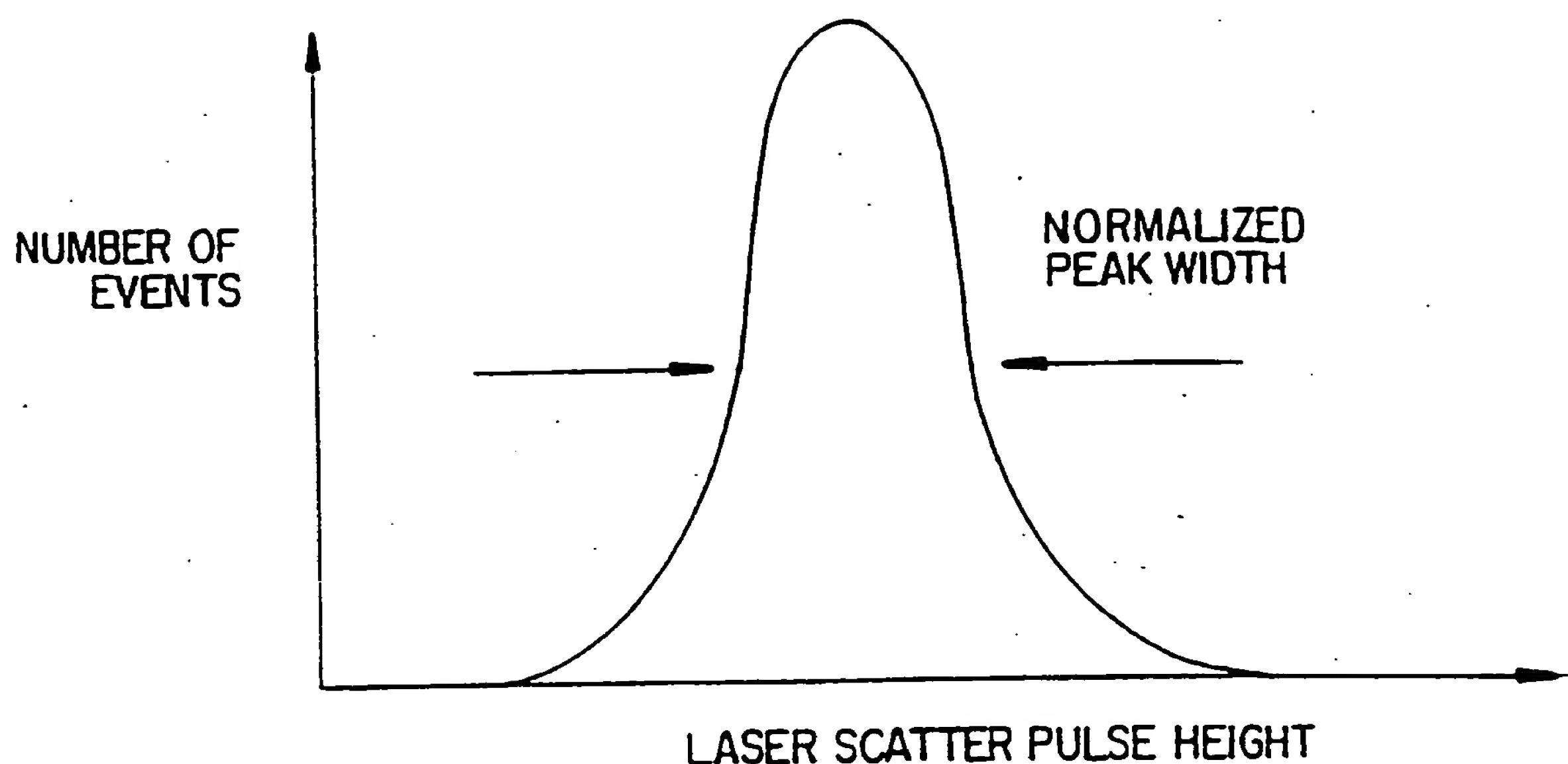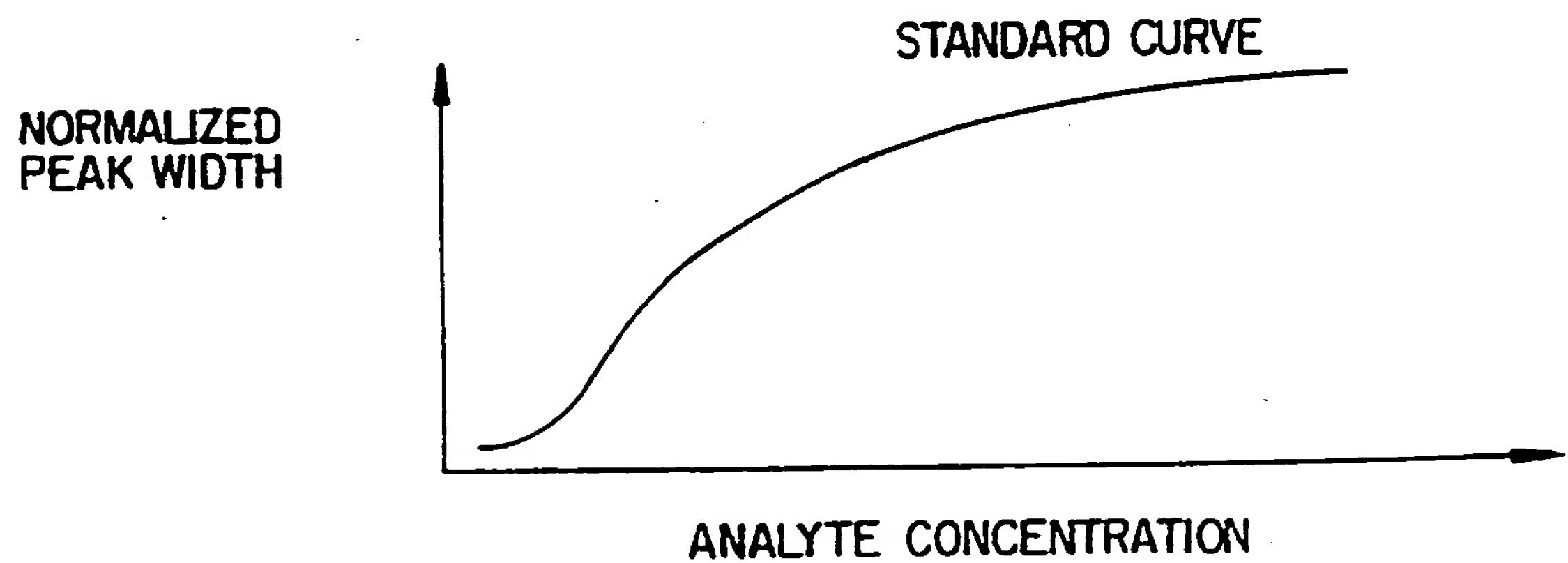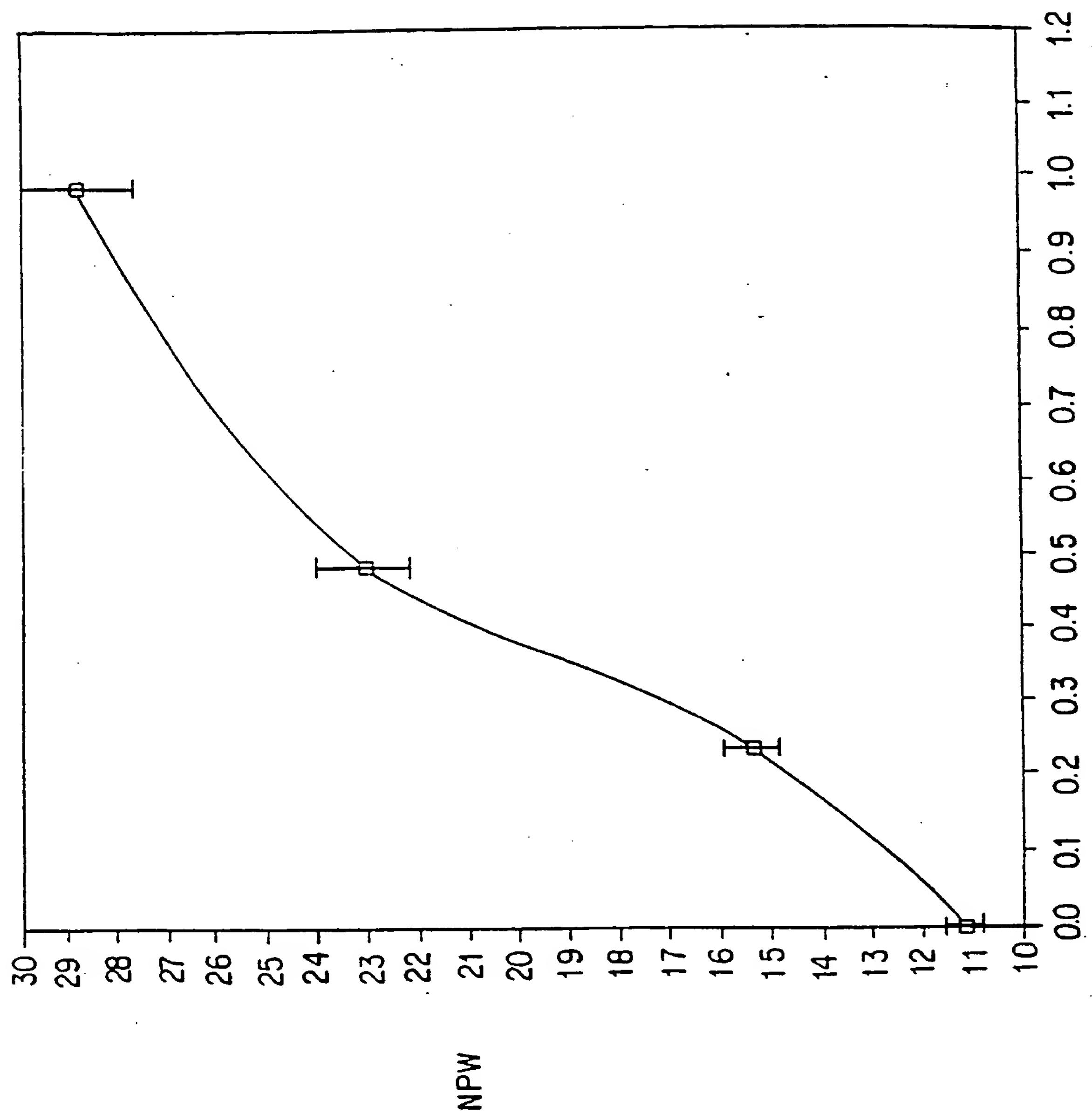
5   laser light.

POLYSTYRENE
SPHERES

BEFORE REACTION

METAL COLLOIDAL PARTICLES

NUMBER OF EVENTS

LIGHT SCATTER PULSE HEIGHT

FIG.1B

POLYSTYRENE
SPHERES

AFTER REACTION

METAL COLLOIDAL PARTICLES

NUMBER OF EVENTS

LIGHT SCATTER PULSE HEIGHT

FIG.1C

POLYSTYRENE
SPHERE

COLLOIDAL
METAL PARTICLE

FIG.1A

FIG. 1D

NUMBER OF
EVENTS

NORMALIZED
PEAK WIDTH

LASER SCATTER PULSE HEIGHT

FIG. 2A

NORMALIZED
PEAK WIDTH

STANDARD CURVE

ANALYTE CONCENTRATION

FIG. 2B

FIG. 3A

FIG. 3B

HISTOGRAM
NPW

T4 MICROGRAM/DL
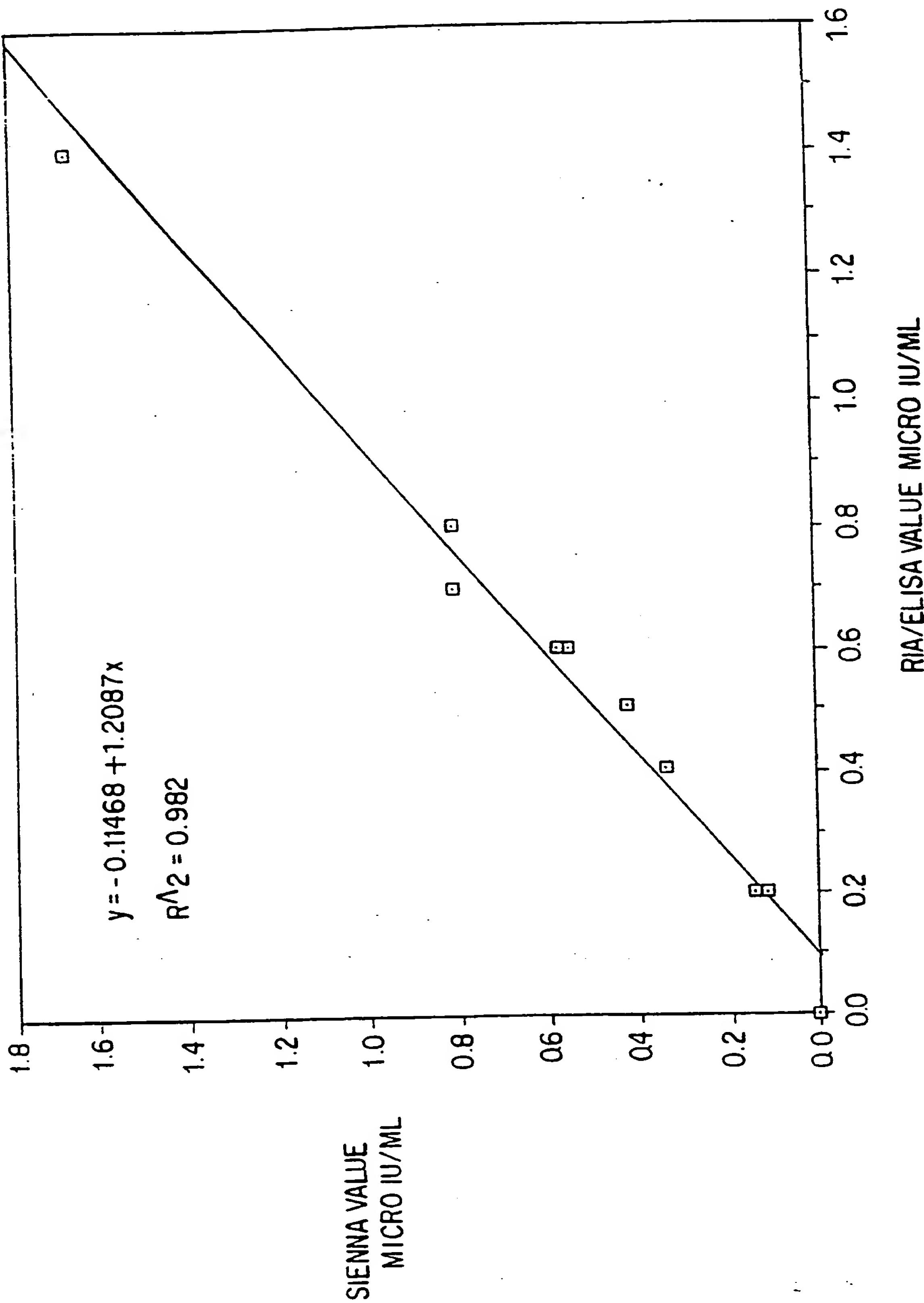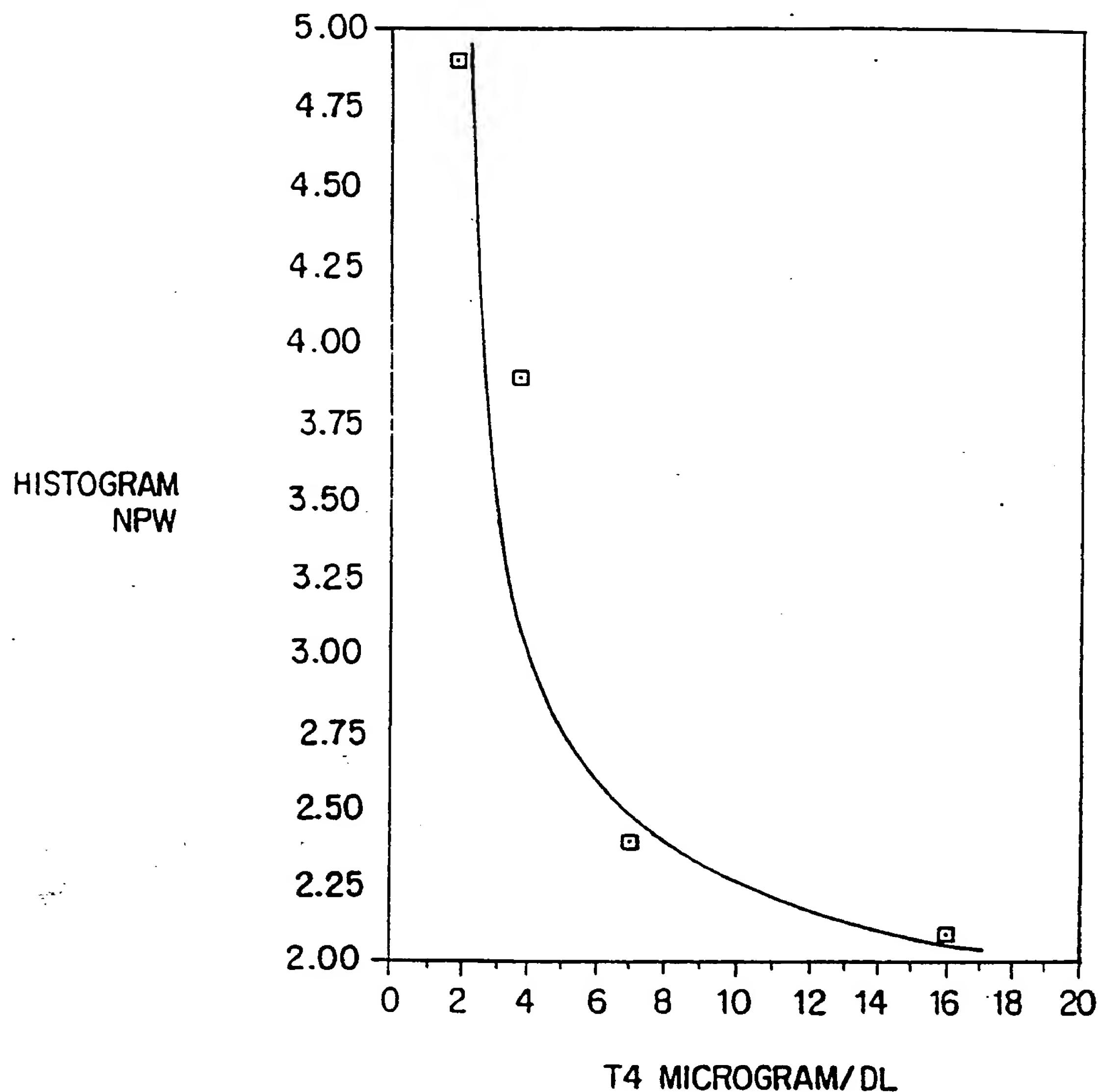
FIG. 4A
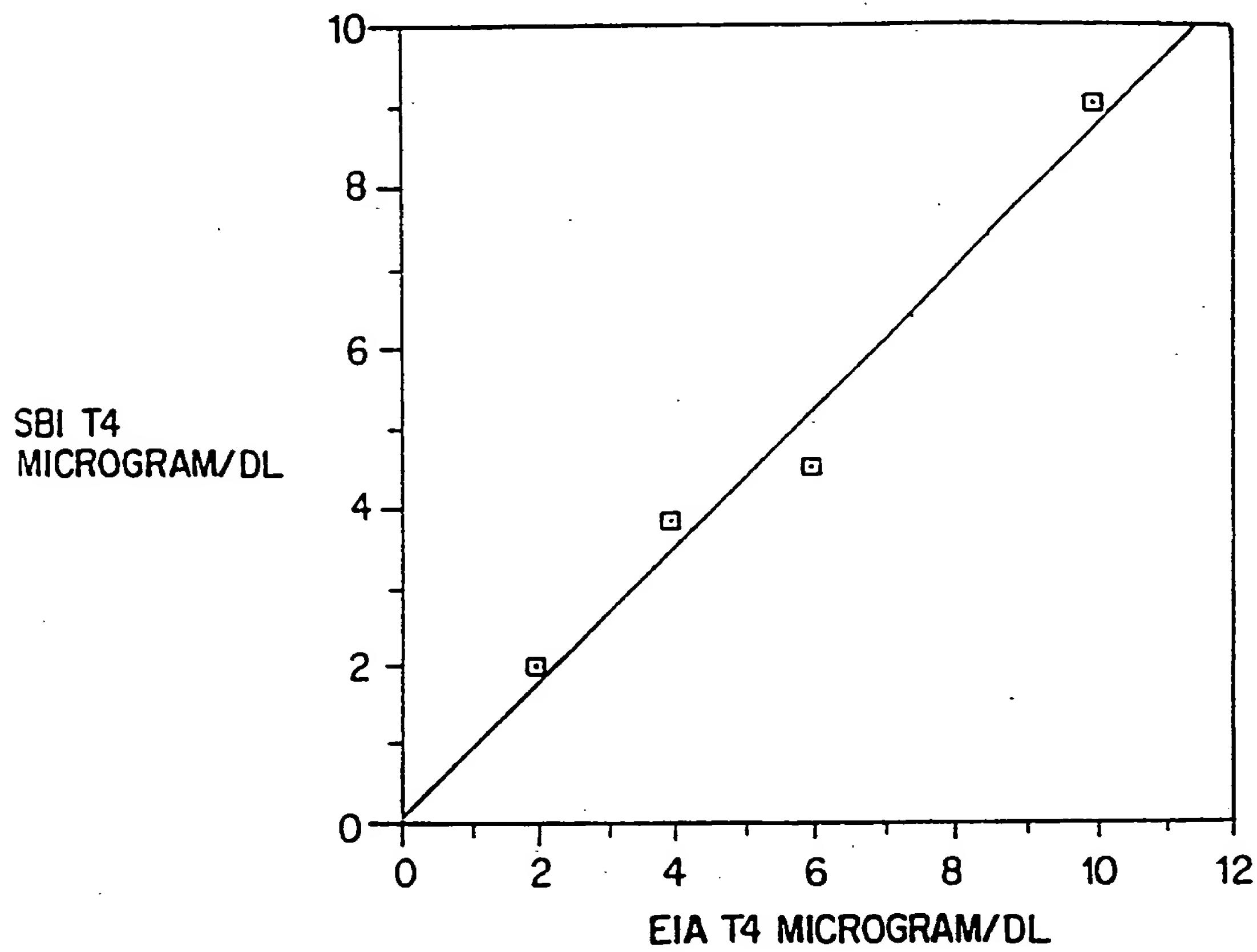
FIG. 4B
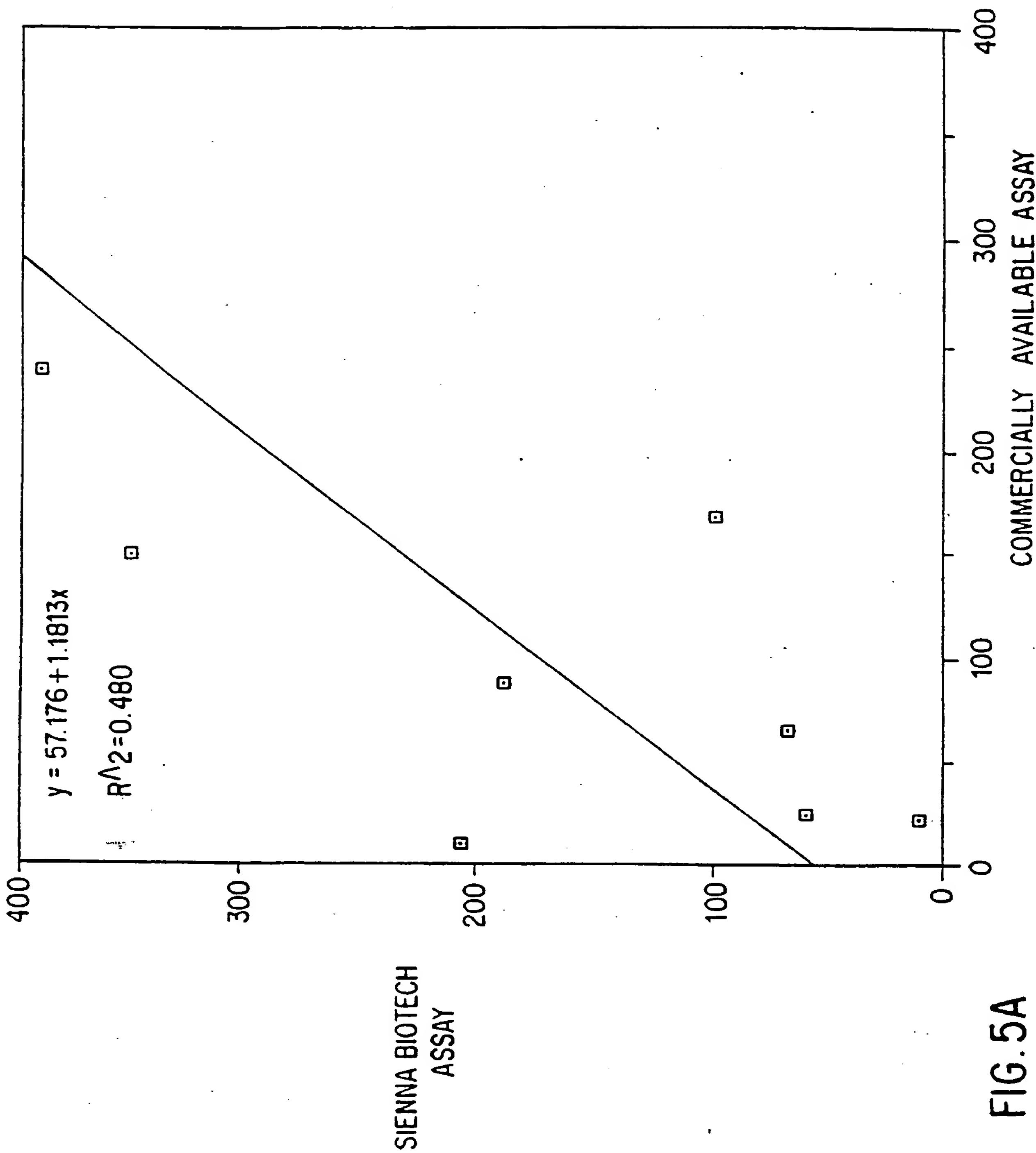
FIG. 5A
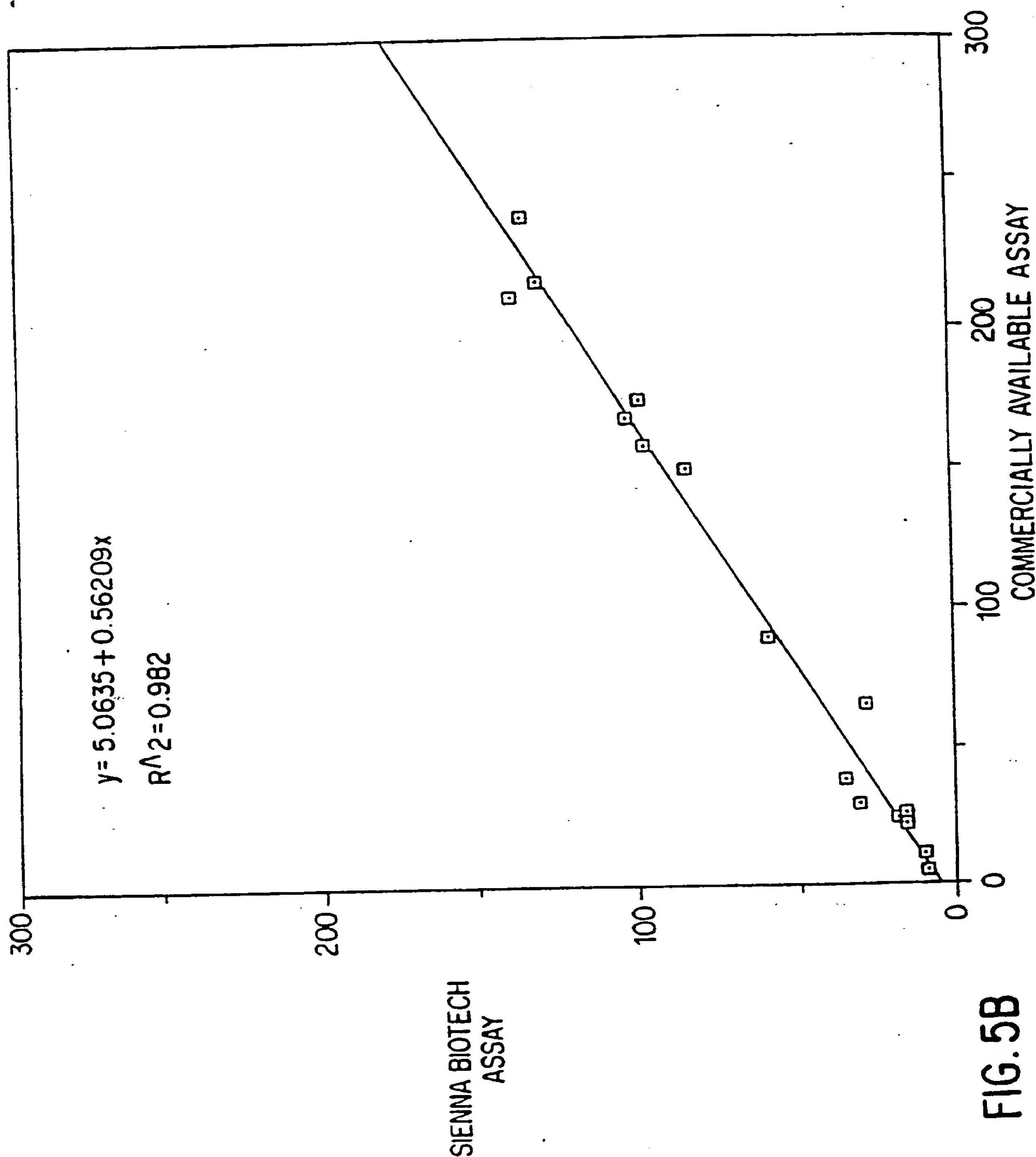
FIG. 5B
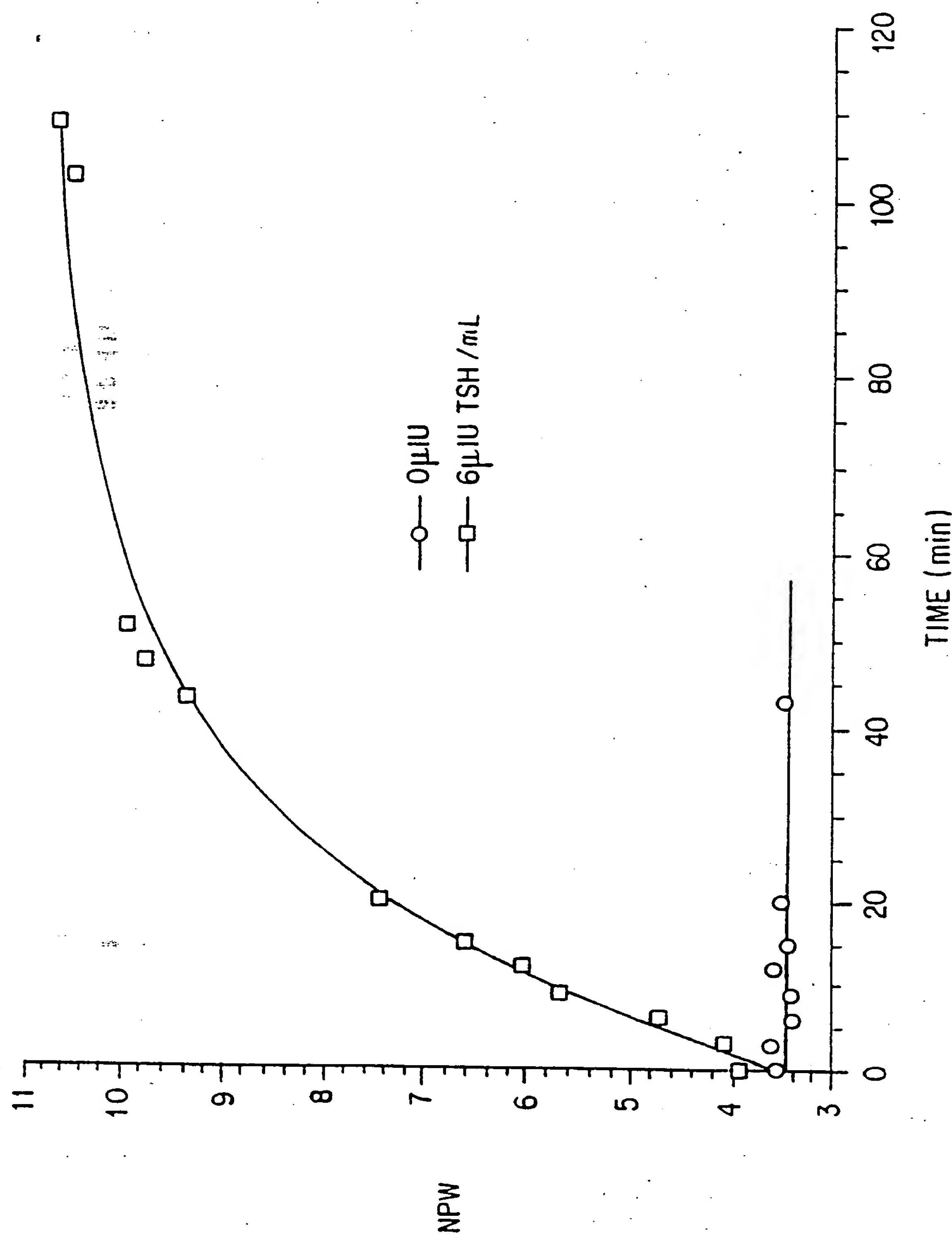
FIG. 6

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 15/14; 21/53, 21/64; 33/544, 33/546, 33/553
US CL :356/336, 337; 435/7.2, 287; 436/172, 525, 528, 533
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 356/336, 337; 435/7.2, 287; 436/172, 525, 528, 533

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
search terms: particle analyzer, flow cytometer, colloidal gold, microsphere, particle, light scatter.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 5,079,172 (HARI ET AL) 07 JANUARY 1992, see column 1, lines 59-68; column 3, lines 43-65; column 7, lines 62-68; column 8, lines 32-39.	32 1-32
X	DUKE SCIENTIFIC CORPORATION, Bulletin 88C, 01 September 1990, "COVASPHERES® REAGENTS", see entire bulletin.	32
X	DUKE SCIENTIFIC CORPORATION, Bulletin 93D, 01 April 1992, "FLUORESCENT MICROSPHERES AND PARTICLES", see entire bulletin.	32

[X] Further documents are listed in the continuation of Box C.	[ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 February 1994	1 4 MAR 1994

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer NANCY J. PARSONS *Jell Warden for*
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLINICAL CHEMISTRY, Volume 31, Number 12, issued 1985, G.C. Saunders et al, "Amplified Flow-Cytometric Separation-Free Fluorescence Immunoassays", pages 2020-2023, see especially pages 2020-2021 and Figure 1A-1C.	1-34
Y	US, A, 4,581,334 (KIRCHANSKI ET AL) 08 APRIL 1986, see column 2, lines39-66; column 8, lines 53-60.	1-34
Y	W.D. ODELL et al, "PRINCIPLES OF COMPETITIVE PROTEIN-BINDING ASSAYS", published 1983 by John Wiley & Sons, see pages 243-254, especially page 244, Figure 1.	22-24,27-29